

REMARKS

Applicant requests reconsideration of the aforementioned application in view of the foregoing amendments and the discussion below. The status of the claims is as follows: Claims 1, 7, 10, 12 and 22 have been amended herein.

The Amendment

Claim 1 was amended to recite that the non-steroidal anti-inflammatory is an arylpropionic acid or a cyclized derivative thereof. Support therefor is in the Specification, for example, page 16, lines 3-5.

Claims 7 was amended to recite at least one hydroxyl group and one or more carboxyl groups and 1 to 4 hydroxyl groups wherein the non-steroidal anti-inflammatory is an arylpropionic acid or a cyclized derivative thereof. Support therefor is in the Specification, for example, page 16, lines 3-5.

Claim 10 was amended to recite that the R-NSAID is an arylpropionic acid or a cyclized derivative thereof and that the compound comprises one or more carboxyl groups and 1 to 4 hydroxyl groups. Support therefor is in the Specification, for example, page 16, lines 3-5.

Claims 12 and 22 were amended in a manner similar to that for Claim 1.

Rejection under 35 U.S.C. §112

Claims 31 and 32 were rejected under 35 U.S.C. 112, first paragraph, as failing to comply with the written description requirement. The Office Action contends that the claim(s) contains subject matter that was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention. The Office Action asserts in particular that the specification doesn't recite the process described in said claim.

Applicant respectfully traverses the above ground of rejection. The paragraph bridging pages 25-26 provides an exemplary synthesis of an R-isomer of an NSAID as depicted in Fig. 1 by way of illustration and not limitation. The specification states that "vicinal hydroxy groups of ascorbic acid **10** are reacted with protecting groups X to give

compound 11. Subsequent reaction of compound 11 with an activated R-isomer of an NSAID, having the carboxylic acid group of the NSAID activated to form the acid chloride, yields compound 12. The protecting groups are removed to give compound 13, namely, an ascorbic acid ester of the R-isomer of an NSAID." This recitation in the specification fully supports the claim language of Claim 31.

Claim 32 is additionally supported in the specification, the paragraph bridging pages 21-22. and 32.

The Office Action indicated that the third page of Manfredini, *et al.*, J. of Bioorganic Med. Chem. Article, was not in the record. Enclosed is a replacement copy of the whole article. Applicant regrets unintentional omission of page 3 from the reference.

Rejection under 35 U.S.C. §102

Claims 1-4, 6, 7, 9, 10, 11 and 15-21 were rejected under 35 U.S.C. 102(e) as being anticipated by Zavitz, *et al.*, Published U.S. Patent Application 20030027867 filed June 28, 2002 (Zavitz). The Office Action characterizes Zavitz as disclosing the C1-C6 alkyl esters of R-NSAIDS as useful for treating a patient with an aids infection.

Zavitz does not disclose or suggest the presently claimed ester compounds. Claim 1 and those claims depending therefrom recite that the esterifying agent comprises 3 to 6 carbon atoms, at least one hydroxyl group and one or more carboxyl groups or 1 to 4 hydroxyl groups or one or more aldehyde groups or a gamma lactone or a delta lactone or an amine or an imine or a lactam. The alkyl esters of Zavitz do not satisfy the definition of esterifying agent of the claims.

Claim 7 recites that the esterifying agent comprises 3 to 6 carbon atoms, at least one hydroxyl group and one or more carboxyl groups and 1 to 4 hydroxyl groups. The alkyl esters of Zavitz do not satisfy the definition of esterifying agent of Claim 7 and those claims dependent therefrom.

Claim 10 recites that the compound comprises one or more carboxyl groups and 1 to 4 hydroxyl groups. The alkyl esters of Zavitz do not satisfy the elements of Claim 10 and those claims dependent therefrom.

Rejection under 35 U.S.C. §103

Claims 1-4, 6, 7, 9, 10, 11 and 15-21 were rejected under paragraph (a) of the above code section as being unpatentable over Wechter, *et al.*, Cancer Research, vol. 60. p. 2203-2208 (2000) (Wechter) in view of Zavitz. Wechter discloses the utility of R-NSAIDS.

Zavitz does not disclose or suggest the presently claimed ester compounds as demonstrated above. The combined teaching of Wechter and Zavitz does not cure this deficiency. Furthermore, the conclusion that, since Zavitz teaches an equivalence of the acid and C1-C6 alkyl esters in treating aids, one skilled in the art would be motivated to convert the free R-NSAID acid of Wechter to the esters of Zavitz to provide pharmaceutically useful, in particular, anti-cancer compounds, is not warranted. The success of such imagined compounds as anti-cancer compounds would not be expected. Thus, even if for the sake of argument the skilled artisan would be so motivated, there would not be any reasonable expectation of success. At most, the situation would be one of obvious to try, which is not the standard for motivation under the statute.

Claims 1-6 and 10-32 were rejected under paragraph (a) of the above code section as being unpatentable over Wechter in view of Manfredini, *et al.*, J. of Med. Chem. (Manfredini). Manfredini's disclosure relates to nipecotic acid, kinurenic acid and diclofenamic acid. These acids are not included in the definition of non-steroidal anti-inflammatory agent of the present claims. Even if for the sake of argument the skilled artisan would be motivated to combine the teachings of Wechter and Manfredini, there would not be any reasonable expectation of success. There is little reason that one skilled in the art would have to conclude that ascorbate esters of Wechter's compounds would be clinically effective. The skilled artisan would not have a likelihood of success. As above, at most, the situation would be one of obvious to try, which is not the standard for motivation under the statute. Furthermore, Manfredini makes no disclosure relevant to other esterifying agents recited in the claims.

Claims 1-6 and 10-32 were rejected under 35 U.S.C. 103(a) as being unpatentable over Zavitz in view of Manfredini. The Office Action asserts that the former discloses the benefit of using the free acid as well as lower alkyl esters of NSAIDS in the R-form in the treatment of AIDS. Because of the disclosure of Manfredini concerning the pharmaceutical

utility of using the ascorbates of certain drugs including an NSAID, argues the Office Action, the ascorbates of the R-NSAIDS of Zavitz would have been obvious to one of ordinary skill in the art.

Even if for the sake of argument the skilled artisan would be motivated to combine the teachings of Wechter and Manfredini, there would not be any reasonable expectation of success. As above, at most, the situation would be one of obvious to try, which is not the standard for motivation under the statute. There is little reason that one skilled in the art would have to equate ascorbate esters and simple C1-C6 alkyl esters as to their effectiveness in treating AIDS. The skilled artisan would not have a likelihood of success. Furthermore, Manfredini makes no disclosure relevant to other esterifying agents recited in the claims.

Claims 1-11 and 15-21 are rejected under 35 U.S.C. 103(a) as being unpatentable over Zavitz in view of Alper, EP 305089 (Alper). Alper discloses a process for the asymmetric production of carboxylic acid esters and/or carboxylic acids. The process comprises reacting a prochiral olefinic function and an alcoholic function or water with carbon monoxide, a single enantiomer of an optically active compound such as menthol, tartaric acid, tartaric acid ester, sugars, proteins and polypeptides, enzymes and chiral phosphines and a catalyst.

The Office Action contends that Alper's disclosure of his method for preparing carboxylic acid esters combined with the teaching of Zavitz results in the presently claimed invention of Claims 1-11 and 15-21. However, one of the esterifying agents specifically claimed in the present application is tartaric acid. Since Alper employs an optically active tartaric acid in his method, one skilled in the art would not reasonably look to Alper for any teaching that is combinable with Zavitz to produce the presently claimed compounds. Furthermore, it would be reasonable for the skilled artisan to conclude that Alper actually teaches away from the present invention. Accordingly, one skilled in the art would not have the requisite motivation for making the combination of teachings of the references suggested in the Office Action.

Claims 1-11 and 15-21 were rejected under 35 U.S.C. 103(a) as being unpatentable over Wechter in view of Zavitz and Alper. The Office Action asserts that Wechter and Zavitz are used to show the desirability of using R-esters of NSAIDS and that Alper shows how to


make them and the optically active esters of NSAIDS with polyhydric alcohols.

For reasons similar to those discussed above with respect to the rejection over Zavitz in view of Alper, the combined teachings of Wechter, Zavitz and Alper do not suggest the presently claimed inventions.

SUMMARY

In summary, the claims 1, 7, 10, 12 and 22 are amended and arguments are submitted to overcome the current rejections. It is believed that claims 1-32 satisfy the requirements of 35 U.S.C. §§112, 102 and 103. Allowance of the above-identified patent application is respectfully requested.

Respectfully submitted,


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Novel Antioxidant Agents Deriving From Molecular Combinations of Vitamins C and E Analogues: 3,4-Dihydroxy-5(*R*)-[2(*R,S*)-(6-hydroxy-2,5,7,8-tetramethylchroman-2(*R,S*)-yl-methyl)-[1,3]dioxolan-4(*S*)-yl]-5*H*-furan-2-one and 3-*O*-Octadecyl Derivatives

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Received 27 January 2000; accepted 31 July 2000

Abstract—Molecular combinations of two antioxidants (i.e., ascorbic acid and the pharmacophore of α -tocopherol), namely the 2,3-dihydroxy-2,3-enono-1,4-lactone and the chromane residues, have been designed and tested for their radical scavenging activities. When evaluated for their capability to inhibit malondialdehyde (MDA) production in rat liver microsomal membranes, the 3,4-dihydroxy-5*R*-2(*R,S*)-(6-hydroxy-2,5,7,8-tetramethylchroman-2(*R,S*)-yl-methyl)-[1,3]dioxolan-4(*S*)-yl]-5*H*-furan-2-one (**11a–d**), exhibited an interesting activity. In particular the 5*R*,2*R*,2*R*,4*S* and 5*R*,2*R*,2*S*,4*S* isomers (**11c,d**) displayed a potent antioxidant effect compared to the respective synthetic α -tocopherol analogue (**5**) and natural α -tocopherol or ascorbic acid, used alone or in combination. Moreover, the mixture of stereoisomers **11a–d** also proved to be effective in preventing damage induced by reperfusion on isolated rabbit heart, in particular at the higher concentration of 300 μ M. In view of these results our study represents a new approach to potential therapeutic agents for applications in pathological events in which a free radical damage is involved. Design, synthesis and preliminary biological activity are discussed. © 2000 Elsevier Science Ltd. All rights reserved.

Introduction

The area of free radical biology and medicine is developing faster since the discovery, in recent years, of the involvement of free radicals in oxidative tissue injury and a growing number of diseases. The production of free radicals and various reactive oxygen species (ROS), derives from oxidative injuries related to pollution, radiation, food constituents and is also a function of the body's normal defence system and metabolic reactions.¹

The involvement of free radicals in the pathology of human diseases (i.e., intestinal diseases, atherosclerosis, reperfusion injuries, cardiac diseases, neurodegeneration, respiratory disorders, inflammation, diabetes, cancer and aging) has been recognized by a number of authors.² Potential therapeutic interventions might include natural antioxidants or synthetic pharmacological agents with antioxidant activity.^{2,3}

The importance of vitamins E and C in disease prevention has become widely recognized and is supported by several clinical and epidemiological studies.⁴ α -tocopherol (vitamin E) provides the most important line of defence against lipid peroxidation of cell membranes, and some of its effects are mediated by interaction with specific and saturable binding sites on cell membranes. However, this activity is strongly dependent on the regeneration of vitamin E by ascorbic acid (vitamin C) and glutathione present in the cytosol. Electron spin resonance studies (ESR) have demonstrated the interaction between vitamin C and E during the free radical mediated oxidative stress, thus indicating the role of ascorbic acid as the terminal small-molecule antioxidant in biological systems.⁵ Therefore, ascorbic acid itself is not able to prevent lipid peroxidation.⁶ It is well known that dietary supplementations of these vitamins are scarcely active, due to their low stability and bioavailability: oxygen-derived free radicals have short half-life and are involved in fast rate reactions (i.e., free radical chain reactions). Thus, the principal requisite that a radical scavenger must possess is to be available, in the

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Table 1. Antioxidant efficiency of the studied compounds **3**, **5**, **11**, **12** and L-ascorbic acid in rat liver microsomal membranes initiated with AAPH^a

Compound	Concentration (μM)	Malondialdehyde inhibition (%)
5	10	15
	25	20
	100	22
3	10	8
	25	20
	100	24
11a,b (cis)	10	20
	25	48
	100	93
11c,d (trans)	2.5	54
	10	77
	25	80
	100	100
12	10	8
	25	18
	100	27
L-ascorbic acid	10	47
	25	91
	100	93

^aMembranes were incubated with 25 mM AAPH for 15 min. Values are the means of three different experiments. Each mean has a standard error less than 10%.

appropriate concentration, at the place where the radical-promoted degradation of the cellular components initiate (i.e., membrane or nucleus and cytoplasm). Up to date, attempts have been made to develop analogues of these vitamins with improved pharmacokinetics (i.e., esters),^{2,7} and a few conjugates between vitamin C and E have been reported.^{8,9} However, the strategy generally employed for the design of these compounds makes use of the more accessible acid hydroxy residues for the conjugation, namely at the 2 and 3 position. Because these functional groups are responsible for the scavenging activity, these compounds act as merely pro-drugs that require activation in the body of the pro-vitamin and do not warrant a

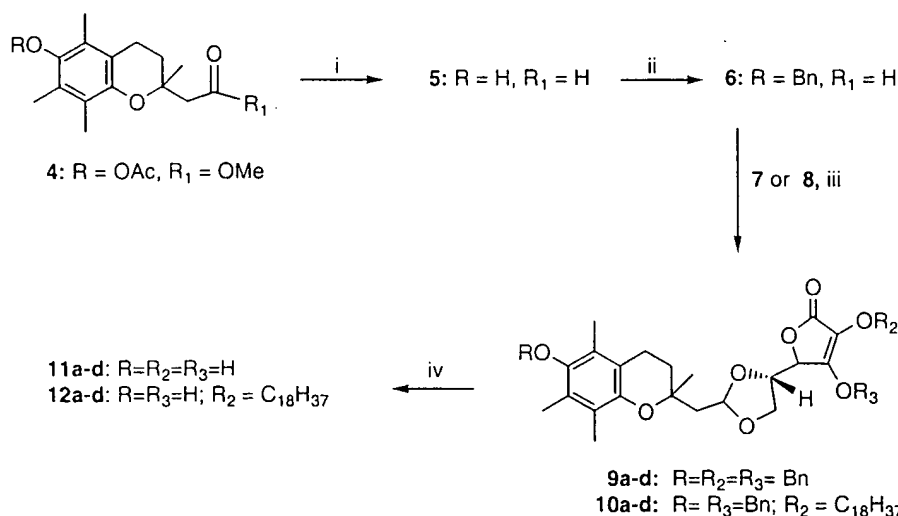
correct delivery of the active moieties to the oxidation site. Taking this into account and continuing our efforts toward the study of radical scavenging agents,^{10,11} we have now designed new analogues of these vitamins in the attempt to overcome the above cited drawbacks. In particular, we have studied molecular combinations of the pharmacophores of the two vitamins (**1**), namely the chromane and the 2,3-dihydroxy-2,3-enono-1,4-lactone rings. The title compounds were synthesized and investigated for their antioxidant activity by evaluation of their capability to inhibit malondialdehyde (MDA) production in rat liver microsomal membranes (Table 1). Moreover, in view of the high relevance of myocardial infarct among cause of death, the compounds (**11a–d**), emerging as promising from the primary antioxidant test were further evaluated for their capability to reduce ischemia-reperfusion damage.

Our study, initiated by a careful examination of the literature, led us to select, as suitable candidates for antioxidant and conjugable moieties, the following molecules: (i) the (*R,S*)-6-hydroxy-(2,5,7,8-tetramethylchroman-2-yl) acetaldehyde (**5**),^{12,13} (ii) the ascorbic acid itself (**2**) and (iii) the 2-*O*-octadecyl-ascorbic acid (**3**), which has shown, together with important antioxidant properties, an increased stability by comparison to ascorbic acid.^{6,14}

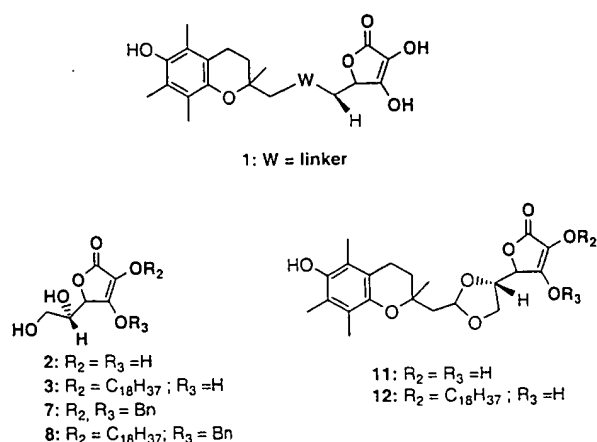
Chemistry

Synthesis

After having evaluated several functionalities, the retrosynthetic analysis suggested us to select an aldehyde on the chromanyl fragment, in order to employ acetalization of the 5- and 6-hydroxy groups on ascorbic acid, for the conjugation of the two fragments. This strategy allowed us to use, for the conjugation process, functional groups of the molecules not involved in the radical scavenging process.



Scheme 1. (i) DIBAL-H, hexane, -70 °C; (ii) benzylbromide, K₂CO₃, DMF, 25 °C; (iii) TsOH, toluene, reflux; (iv) H₂, Pd/C, EtOAc, 25 °C.



Title compounds were synthesized (Scheme 1) in two simple steps from compound **6**, prepared by reduction of (*R,S*)-6-ethyl-(2,5,7,8-tetramethylchroman-2-yl)acetate (**4**)¹² with diisobutylaluminum hydride (DIBAH) to give the corresponding aldehyde **5**, which was protected at the hydroxy function in position 6 by benzylation to afford **6**. Compound **6** was next acetalized, in the presence of *p*-toluenesulfonic acid (TsOH), as catalyst, with 2,3-di-*O*-benzyl-ascorbic acid (**7**)¹⁴ or with 2-*O*-octadecyl-3-*O*-benzyl-ascorbic acid (**8**).¹⁵ Acetalisation proceeded smoothly to give in a 1:1:1:1 mixture of the four expected diastereoisomers, namely 3,4-dibenzyloxy-5*R*-2(*R,S*)-(6-hydroxy-2,5,7,8-tetramethylchroman-2(*R,S*)-yl-methyl)-1,3]dioxolan-4*S*-yl]-5*H*-furan-2-one (**9a–d**) and 4-*O*-benzyl-oxy-5*R*-2(*R,S*)-(6-benzyloxy-2,5,7,8-tetramethylchroman-2(*R,S*)-yl-methyl)-1,3]dioxolan-4*S*-yl]-3-octadecyloxy-5*H*-furan-2-one (**10a–d**). Compounds **10a–d** could be isolated only as inseparable mixtures of the two couple of diastereoisomers: **a,b** and **c,d**. The composition of the mixture was estimated, by ¹H NMR analysis, as the relative intensity of the H-C4 proton, on the furanone ring, in the **a,b/c,d** isomers. These data were also corroborated by NOESY and NOE-difference experiments, indicating a shielding effect, of the methyl at C2 of the chromanyl moiety on the H-C5 at the furanone ring, in the case of the **c,d** isomers. In particular the 5*R*,2*S*,2*S*,4*S* and 5*R*,2*S*,2*R*,4*S* (**a,b**) and the 5*R*,2*R*,2*R*,4*S* and 5*R*,2*R*,2*S*,4*S* (**c,d**) isomers may be considered as *cis* and *trans*

isomers respectively, with regards to the relative stereochemistry of the protons at the C-2 and C-4 of the dioxolane ring.

Protective benzyl groups, at positions 3 and/or 2 on the ascorbic acid moiety and at position 6 on the chromanyl residue, were concomitantly removed by hydrogenation in the presence of 5% Pd/C to give compounds **11a–d** and **12a–d** (Fig. 1).

Structure assignments were also conducted on the base of our previous findings¹⁶ and of quantum-mechanics calculations. Briefly, the structure of **a,b** and **c,d** isomers of compound **11** were optimized and subjected to a systematic conformational search. The structures having a maximum energy difference of 20 kcal/mol were further optimized until convergence. The geometry was optimized both with a molecular mechanics force field (MAXI-MIN2) and with a semi-empirical molecular orbital method (AM1). The conformations having closer distances between C5 proton at the furanone ring and C3 proton and C2 methyl at the chromanyl residue were studied with ab initio Hartree–Fock methods. Molecular modeling confirmed the upfield shift of about 0.06 ppm observed in the **11c,d** (*trans*) isomers for the C-5 proton at the furanone ring, that might be explained on the base of a preferred conformation in which the C-5 proton was in the shielding region of the chromanyl ring. In particular, the **11c,d** isomers show a closer contact of the C5 proton at the furanone ring with the methyl at the C2 of the chromanyl residue, about 4.19 Å, versus 6.43 Å, as compared to the corresponding **11a,b** (*cis*) isomers. Although molecular modeling calculations have permitted the structure assignments for the **a,b** and **c,d** couple of isomers, because no crystalline structure suitable for X-ray spectroscopy could be obtained, it was impossible to assign the absolute configuration of the single diastereoisomers.

Biology

Prevention of lipid peroxidation

The above prepared compounds were evaluated for their ability to inhibit peroxyl radical dependent lipo-

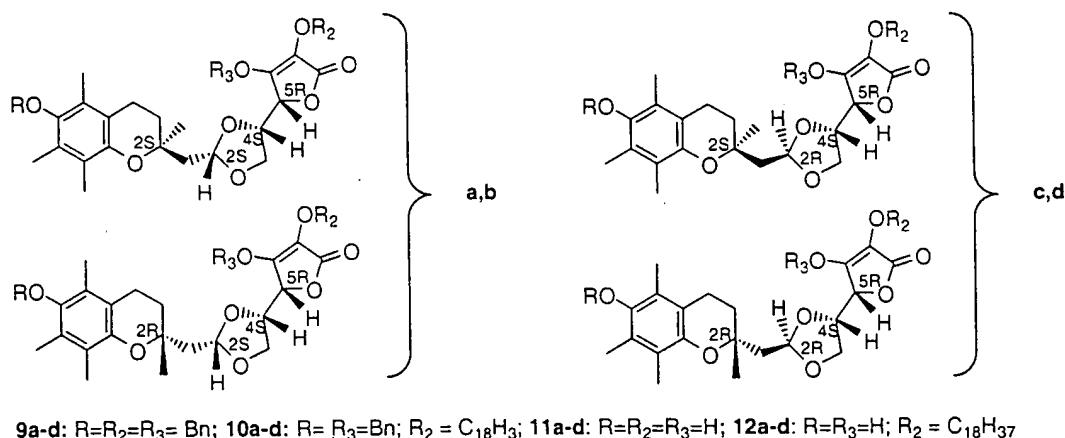


Figure 1. Absolute configuration of stereogenic centers of compounds 9–12.

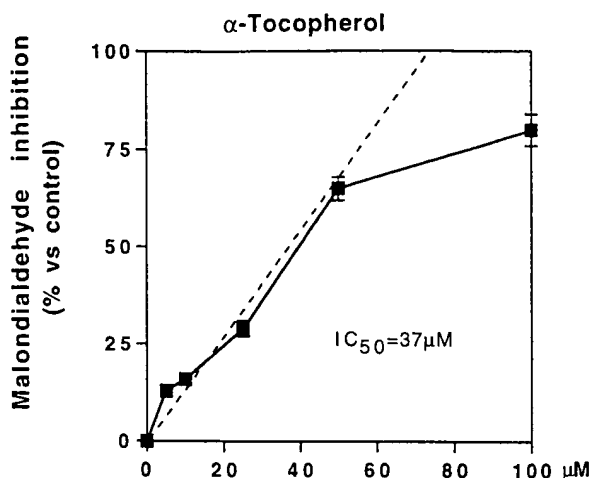


Figure 2. Inhibition of AAPH-induced malondialdehyde production by different concentrations of α -tocopherol in rat liver microsomal membranes. Values are the means \pm SEM of 10 different experiments. Each mean has a standard error less than 10%.

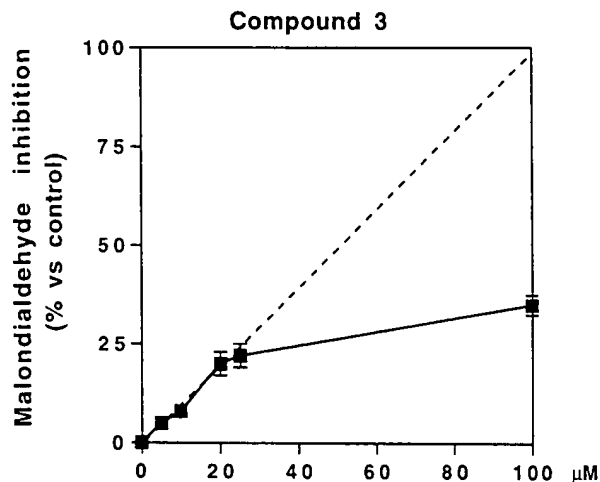


Figure 4. Inhibition of AAPH-induced malondialdehyde production by different concentrations of compound 3, in rat liver microsomal membranes. Values are the means \pm SEM of 10 different experiments. Each mean has a standard error less than 10%.

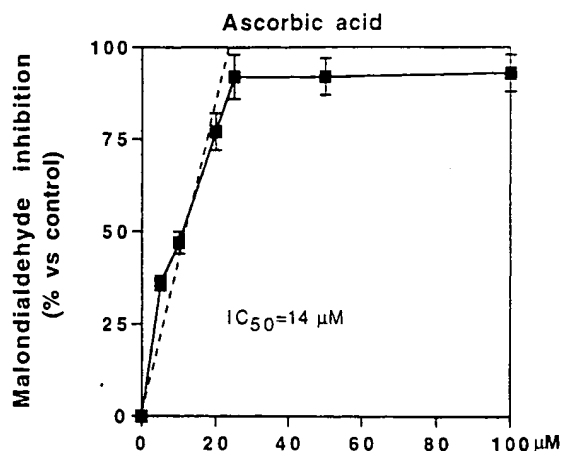


Figure 3. Inhibition of AAPH-induced malondialdehyde production by different concentrations of L-ascorbic acid in rat liver microsomal membranes. Values are the means \pm SEM of 10 different experiments. Each mean has a standard error less than 10%.

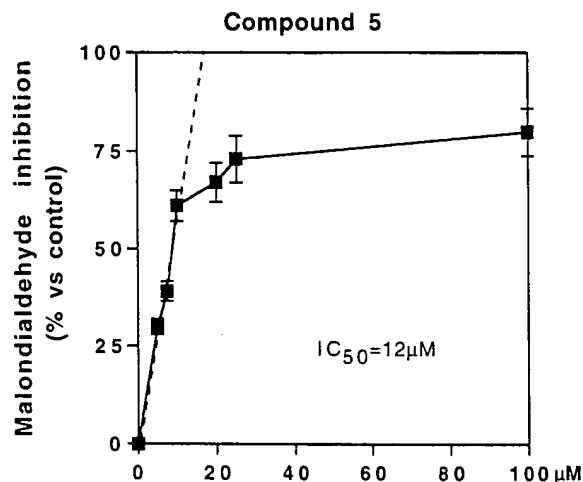


Figure 5. Inhibition of AAPH-induced malondialdehyde production by different concentrations of compound 5, in rat liver microsomal membranes. Values are the means \pm SEM of 10 different experiments. Each mean has a standard error less than 10%.

peroxidation in rat liver microsomal membranes. Microsomes were prepared from Wistar rats as previously reported by some of us¹⁷ and microsomal proteins were determined by the Bio-Rad method. Lipid peroxidation was initiated by 2,2'-azobis(2-amidinopropane)-dihydrochloride (AAPH), which produces peroxy radicals by thermal decomposition. Reactions were carried out at 37°C with 25 mM AAPH for 15 min and lipid peroxidation was measured by the appearance of malondialdehyde (MDA) formation.¹⁸ All compounds were used at the concentration of 5 μ M. Similar results were also obtained using antioxidant concentrations ranging from 2.5 to 100 μ M (data not shown).

In the absence of antioxidants, the formation of MDA in microsomal membranes treated with 25 mM AAPH for 15 min, was 5.40 ± 0.5 nmols/mg protein. Under

these conditions, we have compared the antioxidant ability of the natural antioxidants, α -tocopherol (Fig. 2) and L-ascorbic acid (Fig. 3) to that of the above described synthetic compounds, including 3 (Fig. 4), 5 (Fig. 5), 11a–d (Fig. 6) and 12a–d (Fig. 7). It is evident that all synthetic compounds were able to act as antioxidants, inhibiting AAPH-induced MDA production. In particular, compounds 11a,b and 11c,d, which are the molecular combination between the α -tocopherol analogue 5 and ascorbic acid, exhibited greater antioxidant efficiency (IC_{50} 12 and 7 μ M) than the natural antioxidants, α -tocopherol (IC_{50} 37 μ M) and ascorbic acid (IC_{50} of 14 μ M). Moreover, this compound was a more powerful antioxidant than the synthetic analogue of ascorbic acid, 3, and comparable to the other synthetic analogue of α -tocopherol, 5.

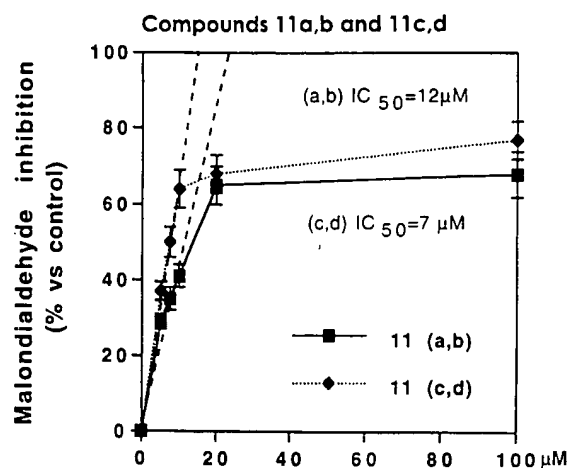


Figure 6. Inhibition of AAPH-induced malondialdehyde production by different concentrations of compounds, 11a,b and 11c,d in rat liver microsomal membranes. Values are the means \pm SEM of 10 different experiments. Each mean has a standard error less than 10%.

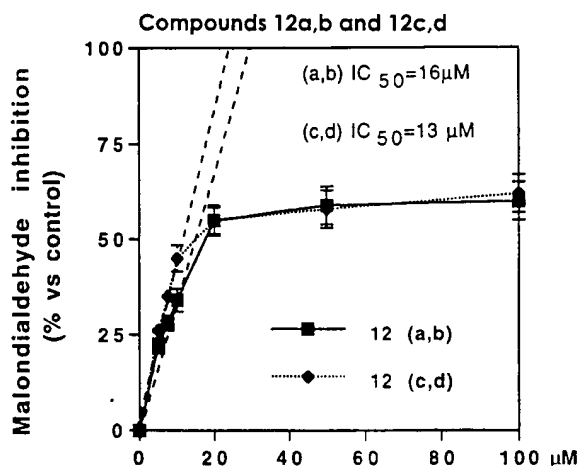


Figure 7. Inhibition of AAPH-induced malondialdehyde production by different concentrations of compounds 12a,b and 12c,d in rat liver microsomal membranes. Values are the means \pm SEM of 10 different experiments. Each mean has a standard error less than 10%.

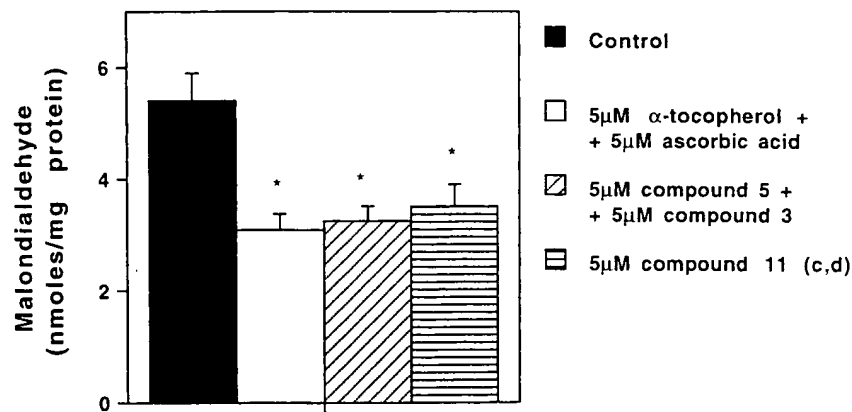


Figure 8. AAPH-induced MDA production in the absence (control) or in the presence of α -tocopherol + ascorbic acid, compound 5 + compound 3 or compounds 11c,d, in rat liver microsomal membranes. Values are the means \pm SEM of three different experiments. The value with asterisk are significantly different from control values ($p < 0.05$).

The configuration at the dioxolane ring and chromane ring also influenced the antioxidants properties of this compound, as shown by the different IC_{50} values of compounds 11a,b and 11c,d. Finally, 11c,d exhibited a greater antioxidant efficiency than the respective 12, confirming that the substitution at position 2 of the ascorbyl residue is important for the antioxidant properties of these compounds, indeed specific enzymes, not present in our preliminary assay, may be responsible of the reported activity of 3.¹⁴

The mechanism at the base of the improved antioxidant efficiency of compounds 11c,d, as compared to other natural and synthetic antioxidants here tested, is currently under investigation. We may advance the hypothesis that the concomitant presence of the ascorbic acid moiety and α -tocopheryl residue may facilitate a radical trapping activity in both hydrophilic and lipophilic compartments by improving the antioxidant potential through concomitant scavenging of lipoperoxyl radicals (preferred by tocopherol) and reactive oxygen species (ROS, preferred by ascorbic acid). Moreover, it may induce increased stability of the compound by limiting its oxidation:¹⁹ the ascorbic acid moiety on α -tocopheryl residue may reduce the production of tocopheroxyl radicals, which are usually formed during the radical scavenging activity and, consequently, may inhibit oxidation induced by these radicals.

In order to verify the influence of possible metabolic transformation, and to compare its activity with that of simple physical mixtures of the corresponding antioxidant moieties, stereoisomers 11c,d were also compared to associations of the natural α -tocopherol and ascorbic acid and of the respective synthetic analogues 5 and 3. As shown in Fig. 7, although 3 was the less active among the test compounds (compare Fig. 4 with Figs 2, 3 and 5–7) no significant differences in AAPH-induced MDA inhibition were found among the compounds 11c,d, and the association of both 3 and 5. This occurrence may be explained on the basis of the high activity displayed by 5 itself (Fig. 5) which represent an unexpected result of this study. The lack of significative

increasing in potencies may also be explained by a reduced mobility of compounds **11c,d** in aqueous phase or within the membrane bilayer with respect to α -tocopherol and ascorbic acid.

Pharmacology

Ischemia-reperfusion in isolated rabbit heart

Ischemic tissues, i.e., heart after myocardial infarct, are vulnerable to damage when rapidly reperfused with oxygen-rich blood in order to reduce the extent of the injury; this occurrence is known as reperfusion damage. The further damage is due to the presence of oxygen-derived free radicals and reactive oxygen species present in the reperfusion medium. Many classes of drugs and

antioxidants such as α -tocopherol offer protection against reperfusion injury and, in view of the relevance of ischemic diseases, there is an increasing interest in the research of new agents. In view of this evidence, we thus decided to investigate the potential of our compounds in preventing damages induced by reperfusion on isolated rabbit heart.

When the perfusion flow rate of electrically paced isovolumic left rabbit heart preparations was reduced from 20 to 1 mL/min for a period of 40 min, a progressive increase in LVEDP values (from 4.1 ± 0.5 to 72.2 ± 6.8 mmHg; $p < 0.001$) occurred suggesting that an ischemic phenomenon was taking place. In fact, ventricular function was impaired since the recovery of post-ischemic LVDP was low and after 20 min of reperfusion, only 38% of the preischemic strength of heart contractility was restored (38.9 ± 4.2 versus 102.5 ± 8.5 mmHg; $p < 0.001$). At this time, CPP considerably augmented over the basal values (from 52.5 ± 5.1 to 96.6 ± 6.3 mmHg; $p < 0.001$) suggesting an increase in coronary vascular resistance owing to the stiffness of the heart (Figs 8 and 9). Furthermore, the partial functional recovery of the hearts during reperfusion was accompanied by a consistent release of CK into the cardiac perfusates. In fact, peak concentration of CK was increased 9.1-fold compared to preischemic values (from 49 ± 6 to 448 ± 40 mU/min/g wet tissue; $p < 0.001$) (Fig. 10).

When the hearts were perfused for 30 min in the pre-ischemic period with graded concentrations of **11a–d** (100–300 μ M), a myocardial protection against mechanical changes due to ischemia-reperfusion was significantly evident at the higher concentration of 300 μ M. In fact, the increase in LVDP values observed in untreated preparations at the end of the ischemic period (from 5.8 ± 0.5 to 34.1 ± 2.5 mmHg; $p < 0.05$) was sig-

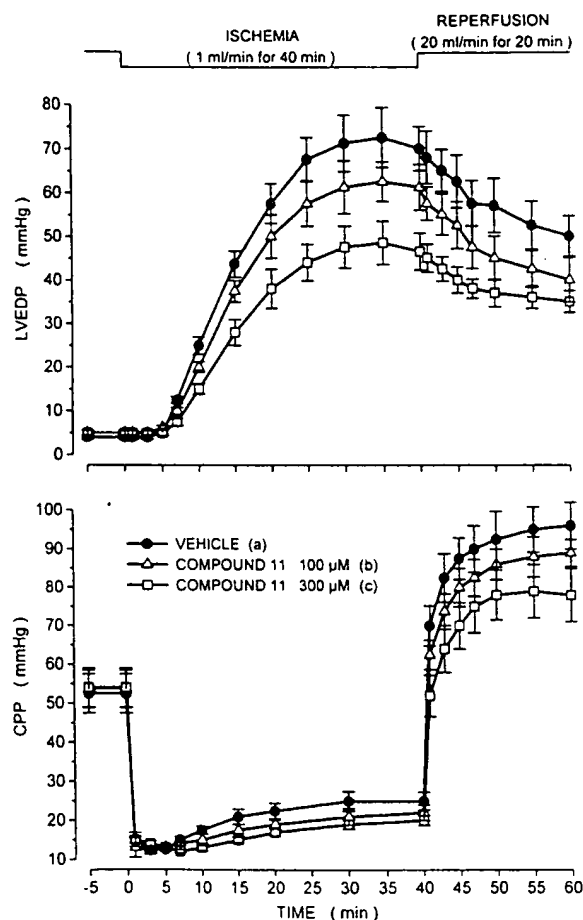


Figure 9. Effects of compounds **11a–d** in perfused rabbit hearts subjected to ischemia-reperfusion: trend of left ventricular end diastolic pressure (LVEDP) and coronary perfusion pressure (CPP). Data are mean \pm SEM of five different heart preparations per group. Compound **11** (100–300 μ M) was perfused through the hearts for a period of 30 min before reduction of coronary flow. The areas under the curve (AUCs) related to LVEDP are: a, 3046 ± 284 ; b, 2577 ± 191 ; c, 2021 ± 177 . The AUCs related to CPP (increase in mmHg over the preischemic values) are: a, 716 ± 83 ; b, 593 ± 49 ; c, 424 ± 23 ; with statistical differences for both LVEDP and CPP: a versus b, $p > 0.05$; a versus c, $p < 0.05$. The AUCs was evaluated according to the trapezoid method: in ordinate, LVEDP or CPP in mmHg; in abscissa, time from 0 to 60 min (for LVEDP) or from 40 to 60 min (for CPP).

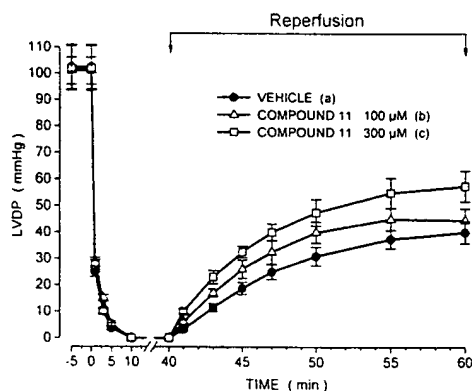


Figure 10. Effects of compounds **11a–d** on left ventricular developed pressure (LVDP) in perfused rabbit heart subjected to ischemia-reperfusion. Data are mean \pm SEM of 5 different heart preparations per group. Compounds **11a–d** (100–300 μ M) were perfused through the hearts for a period of 30 min before reduction of coronary flow. The areas under the curve (AUCs) related to LVDP are: a, 593 ± 42 ; b, 672 ± 57 ; c, 835 ± 52 ; with statistical differences for LVDP: a versus b, $p > 0.05$; a versus c, $p < 0.05$. The AUCs was evaluated according to the trapezoid method: in ordinate, LVDP in mmHg; in abscissa, time from 40 to 60 min.

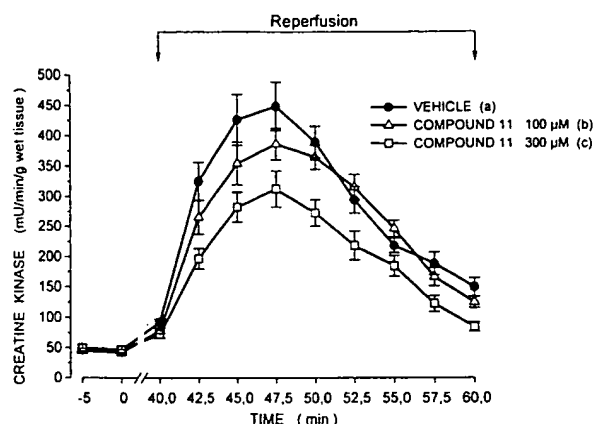


Figure 11. Kinetic profile of creatine kinase (CK) activity released in perfusates of rabbit hearts subjected to ischemia-reperfusion. Data are mean \pm SEM of five different heart preparations per group. Compound **11a–d** (100–300 μ M) was perfused through the hearts for a period of 30 min before reduction of coronary flow. The areas under the curve (AUCs) related to CK are: *a*, 5081 ± 408 ; *b*, 4553 ± 254 ; *c*, 3220 ± 272 ; with statistical differences for CK: *a* versus *b*, $p>0.05$; *a* versus *c*, $p<0.01$. The AUCs was evaluated according to the trapezoid method: in ordinate, CK in mU/min/g wet tissue; in abscissa, time from 40 to 60 min.

nificantly less in **11a–d** in infused hearts and at the end of reperfusion period LVDP reached 57% of the pre-ischemic values (57.5 ± 5.8 versus 101.4 ± 9.3 mmHg; $p<0.01$). At the same time, the increase in CPP in untreated hearts was diminished (from 96.6 ± 6.3 to 77.5 ± 6.9 mmHg; $p<0.05$) indicating a reduction of coronary vascular resistance (Figs 9 and 10). In keeping with these results, the kinetic profile of CK released in the cardiac effluent was significantly different from that observed in untreated preparations. At the peak of the concentration, CK was increased 6.2-fold (from 50 ± 5 to 312 ± 30 mU/min/g wet tissue; $p<0.001$) (Fig. 11).

Stability

In order to investigate if the observed activity may be related to a possible hydrolysis of our molecules, stability of the mixture of stereoisomers **11a–d** was evaluated in water at 37°C, at pH ranging from 3 to 8. Compounds **11a–d** proved stable, with half-lives ranging from 1 to 3 days (Table 2). The obtained half-lives confirm that they act per se and not after transformation into aldehyde **5** and L-ascorbic acid.

Table 2. In vitro hydrolysis of stereoisomers **11a–d** at different pH; $t_{1/2}$ 37°C

Compound	Product	In vitro hydrolysis	
		pH	$t_{1/2}$ 37°C. h ^a
11a–d	5. ascorbate	3	24
		5	38
		7	72
		8	60

^aHPLC analysis; $t_{1/2}$ is the time required for 50% hydrolysis to the corresponding **5** and ascorbic acid.

In conclusion, some new molecules, based on the concept of the molecular combination of cooperative antioxidants, have been prepared. In a peroxy radical-dependent lipoperoxidation assay, these compounds were all inhibitors of MDA formation. However, the synthetic compounds **11c,d**, which are the 5*R*,2*R*,2*R*,4*S* and 5*R*,2*R*,2*S*,4*S* isomers of the molecular combination of an α -tocopherol-analogue and L-ascorbic acid, resulted in interesting antioxidants with a potency comparable, if not slightly superior, to the respective synthetic α -tocopherol analogue (**5**) and natural α -tocopherol or L-ascorbic acid, used alone or in combination. Moreover, the major advantages of this compound derives from its capability to trap free radicals simultaneously in both hydrophilic and lipophilic environments. In view of these data **11a–d** were also investigated for their ability to prevent damage induced by reperfusion on isolated rabbit heart resulting effective at the higher concentration of 300 μ M. Taken together, these results are of significance for possible therapeutic applications in pathological events in which free radical damage is involved and for the possible extension of our approach to other synergistic antioxidants.

Experimental

Materials

Compounds **4**,¹³ **7**¹⁵ and **8**¹⁴ were prepared following and adapting reported procedures.

General

Melting points were determined by a Kofler apparatus and are uncorrected. Reaction courses were routinely monitored by thin layer chromatography (TLC) on silica gel precoated Durasil-25 UV₂₅₄ Machery–Nagel plates with detection under 254 nm UV lamp and/or by spraying the plates with 10% H₂SO₄/MeOH and/or with 5% KMnO₄/H₂O solutions and heating. Nuclear magnetic resonance (¹H NMR) spectra were determined in DMSO-*d*₆, CDCl₃ solutions with a Bruker AC-200 spectrometer and chemical shift are presented in ppm from internal tetramethylsilane as a standard. Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) were obtained using the HPG2025A mass spectrometer. HPLC analysis was performed on Beckman System Gold with a Beckman ultrasphere ODS column (5 mm; 4.6 \times 250 mm). Analytical determinations were carried out by two solvent systems: (method 1) A = water, B = acetonitrile, a linear gradient was initiated after injection of a sample and run from 100% A to 100% B in a 25 min. All compounds showed a purity higher than 99% following analytical HPLC monitored at 220 and 254 nm. Column chromatography was performed with Machery–Nagel 70–230 mesh silica gel. Purity of final compounds was also assessed by high resolution mass and combustion analyses. Where not differently stated, microanalyses were in agreement with calculated values (sd \pm 0.4%).

(*R,S*)-6-Hydroxy-(2,5,7,8-tetramethylchroman-2-yl)-acetaldehyde (5). A solution of 6-ethyl-(2,5,7,8-tetramethylchroman-2-yl)acetate (**4**) (2.5 g, 7.8 mmol) in 130 mL of anhydrous hexane, was cooled under argon atmosphere, in a dry ice/ethanol bath to -70°C . To the resulting suspension was added, dropwise, a solution of diisobutylaluminum hydride in hexane (25%, 13 mL, 23.4 mmol). The reaction was monitored by TLC (hexane:Et₂O 7:3). After 15 min, the reaction was quenched at -70°C with methanol and water, and worked-up with ether, washed with 2M HCl, dried over anhydrous Na₂SO₄ and the solvent was removed under reduced pressure. The crude product was purified by silica gel chromatography (hexane:Et₂O 7:3) to give 1.55 g of white solid; yield 82%. Mp $82\text{--}85^{\circ}\text{C}$ (Et₂O/hexane). ¹H NMR (CDCl₃): δ 1.32 (s, 3H, CH₃-C2); 1.79–1.87 (m, 4H, ArCH₂CH₂); 2.03, 2.05, 2.09 (3s, 9H, 3 CH₃ Ar); 2.4–2.7 (m, 4H, ArCH₂CH₂, CH₂COH); 9.85 (dd, 1H, $J=6$ and 2.4 Hz, CHO). MALDI-TOF MS: m/z 248.7 (M)⁺, C₁₅H₂₀O₃ requires 248.03. Anal. C, H.

(*R,S*)-6-Benzoyloxy-(2,5,7,8-tetramethylchroman-2-yl)-acetaldehyde (6). A mixture of **5** (2.2 g, 8.8 mmol), anhydrous K₂CO₃ (1.28 g, 9.2 mmol), benzyl bromide (1.2 mL, 10.8 mmol), and 20 mL of anhydrous DMF was stirred for 22 h at room temperature. The obtained suspension was poured into H₂O, worked-up with ether, dried over anhydrous Na₂SO₄ and the solvent was removed under reduced pressure. The crude product was purified by silica gel chromatography (hexane:Et₂O 9:1) to give 1.3 g of yellow oil; yield 43%. ¹H NMR (CDCl₃): δ 1.32 (s, 3H, CH₃-C2); 1.79–1.87 (m, 4H, ArCH₂CH₂); 2.03, 2.05, 2.09 (3s, 9H, 3 CH₃ Ar); 2.48–2.61 (m, 4H, ArCH₂CH₂, CH₂COH); 4.73 (s, 2H, CH₂Ph); 7.40–7.56 (m, 5H, Ph); 9.85 (dd, 1H, CHO, $J=6$ and 2.4 Hz). MALDI-TOF MS: m/z 338.7 (M)⁺, C₂₂H₂₆O₃ requires 338.44.

3,4-Dibenzoyloxy-5*R*-2*S*-(6-benzoyloxy-2,5,7,8-tetramethylchroman-2(*R* and *S*)-yl-methyl)-1,3]dioxolan-4*S*-yl]-5*H*-furan-2-one (9a,b) and 3,4-dibenzoyloxy-5*R*-2*R*-(6-benzoyloxy-2,5,7,8-tetramethylchroman-2(*R* and *S*)-yl-methyl)-1,3]dioxolan-4*S*-yl]-5*H*-furan-2-one (9c,d). A mixture of 2,3-*O*-benzyl-ascorbic acid (**7**) (1.2 g, 3.3 mmol), **6** (2.3 g, 6.8 mmol), *p*-toluenesulfonic acid (150 mg, 0.78 mmol) in 90 mL of benzene, was refluxed in a Dean-Stark apparatus for 8 h. The reaction was then poured into H₂O, worked-up with EtOAc, dried over anhydrous Na₂SO₄ and the solvent was removed under reduced pressure, to give a 1:1:1:1 mixture of the four diastereoisomers (HPLC analysis). The crude product was purified by silica gel chromatography (hexane: Et₂O 6:4) to give compounds **9a–d**, overall yield 55%.

9a oil; 230 mg, yield 10%; TLC (hexane:Et₂O 6:4, 0.35); ¹H NMR (CDCl₃): δ 1.21 (s, 3H, CH₃-C2 benzopyran); 1.7 (m, 2H, ArCH₂CH₂ benzopyran); 1.9 (dd, 2H, CH₂, $J=5$ Hz, $J=4.4$); 2.01, 2.08, 2.13 (3s, 9H, 3 CH₃ Ar); 2.5 (t, 2H, ArCH₂CH₂); 3.6–3.95 (m, 2H, H-C5 dioxolane); 4.2 (m, 1H, H-C4 dioxolane); 4.49–4.50 (d, 1H, H-C5 furanone, $J=2.4$ Hz); 4.47 (d, 2H, CH₂-Ph, benzopyran); 5.01 (d, 2H, CH₂-Ph at C3 furanone); 5.09 (m,

1H, H-C2 dioxolane); 5.14 (s, 2H, CH₂-Ph at C4 furanone); 7.1–7.5 (m, 15H, Ph). MALDI-TOF MS: m/z 676.9 (M)⁺, C₄₂H₄₄O₈ requires 676.79.

9b oil; 320 mg, yield 14%; TLC (hexane:Et₂O 6:4, 0.34); ¹H NMR (CDCl₃): δ 1.21 (s, 3H, CH₃-C2 benzopyran); 1.7 (m, 2H, ArCH₂CH₂); 1.9 (dd, 2H, CH₂, $J=5$ Hz, $J=4.4$); 2.01, 2.08, 2.13 (3s, 9H, 3 CH₃ Ar); 2.5 (t, 2H, ArCH₂CH₂); 3.6–3.95 (m, 2H, H-C5 dioxolane); 4.2 (m, 1H, H-C4 dioxolane); 4.47–4.49 (d, 1H, H-C5 furanone, $J=4$ Hz); 4.46 (d, 2H, CH₂-Ph, benzopyran); 5.01 (d, 2H, CH₂-Ph at C3 furanone); 5.09 (m, 1H, H-C2 dioxolane); 5.13 (s, 2H, CH₂-Ph at C4 furanone); 7.1–7.5 (m, 15H, Ph). MALDI-TOF MS: m/z 677.8 (M+H)⁺, C₄₂H₄₄O₈ requires 676.79.

9c foam; 280 mg, yield 12%; TLC (hexane:Et₂O 6:4, 0.32); ¹H NMR (CDCl₃): δ 1.21 (s, 3H, CH₃-C2 benzopyran); 1.7 (m, 2H, ArCH₂CH₂); 1.9 (dd, 2H, CH₂, $J=5$ Hz, $J=4.4$); 2.01, 2.08, 2.13 (3s, 9H, 3 CH₃ Ar); 2.5 (t, 2H, ArCH₂CH₂); 3.6–3.95 (m, 2H, H-C5 dioxolane); 4.2 (m, 1H, H-C4 dioxolane); 4.48–4.50 (d, 1H, H-C5 furanone, $J=3.4$ Hz); 4.6 (d, 2H, CH₂-Ph benzopyran); 5.01 (d, 2H, CH₂-Ph at C3 furanone); 5.10 (m, 1H, H-C2 dioxolane); 5.14 (s, 2H, CH₂-Ph C4 furanone); 7.1–7.5 (m, 15H, Ph). MALDI-TOF MS: m/z 676.8 (M)⁺, C₄₂H₄₄O₈ requires 676.79.

9d foam; 370 mg, yield 16%; TLC (hexane:Et₂O 6:4, 0.30); ¹H NMR (CDCl₃): δ 1.21 (s, 3H, CH₃-C2 benzopyran); 1.6–1.8 (m, 2H, ArCH₂CH₂); 1.9 (dd, 2H, CH₂, $J=5$ Hz, $J=4.4$); 2.01, 2.08, 2.13 (3s, 9H, 3 CH₃ Ar); 2.5 (t, 2H, ArCH₂CH₂); 3.6–3.95 (m, 2H, H-C5 dioxolane); 4.2 (m, 1H, H-C4 dioxolane); 4.50–4.52 (d, 1H, H-C5 furanone, $J=3.2$ Hz); 4.6 (d, 2H, CH₂-Ph benzopyran); 5.01 (d, 2H, CH₂-Ph at C3 furanone); 5.09 (m, 1H, H-C2 dioxolane); 5.14 (s, 2H, CH₂-Ph at C4 dioxolane); 7.1–7.5 (m, 15H, Ph). MALDI-TOF MS: m/z 676.9 (M)⁺, C₄₂H₄₄O₈ requires 676.79.

3,4-Dihydroxy-5*R*-2*S*-(6-hydroxy-2,5,7,8-tetramethylchroman-2(*R* and *S*)-yl-methyl)-1,3]dioxolan-4*S*-yl]-5*H*-furan-2-one (11a,b) and 3,4-dihydroxy-5*R*-2*R*-(6-hydroxy-2,5,7,8-tetramethylchroman-2(*R,S*)-yl-methyl)-1,3]dioxolan-4*S*-yl]-5*H*-furan-2-one (11c,d). The four diastereoisomers were deprotected by the following general procedure: each of the above obtained **9a–d** (200 mg, 0.29 mmol each-one) were dissolved in EtOAc (10 mL) and hydrogenated in presence of 10% Pd/C (50 mg) for 3 h, at atmospheric pressure. The catalyst was then removed by filtration, and the filtrate was concentrated under reduced pressure. The residue was purified by silica gel chromatography (CH₂Cl₂:MeOH 9:1) to give the expected **11a–d** in good yield (90–93%).

11a oil; 106 mg, yield 90%; ¹H NMR (DMSO-*d*₆): δ 1.21 (s, 3H, CH₃-C2 benzopyran); 1.7 (m, 2H, ArCH₂CH₂); 1.85 (dd, 2H, CH₂, $J=4$ Hz, $J=4.4$); 1.95, 2.0, 2.03 (3s, 9H, 3 CH₃ Ar); 2.49 (t, 2H, ArCH₂CH₂); 3.9–4.1 (m, 2H, H-C5 dioxolane); 4.24 (m, 1H, H-C4 dioxolane); 4.79 (d, 1H, H-C5 furanone, $J=4$ Hz); 5.02 (t, 1H, H-C2 dioxolane); 7.40 (s, 1H, OH 6-benzo-

pyran); 8.5 (sbr, 1H, OH at C3 furanone); 11.35 (br, 1H, OH, at C4 furanone). MALDI-TOF MS: m/z 406.8 (M)⁺, C₂₁H₂₆O₈ requires 406.4. Anal. C, H.

11b oil; 108 mg, yield 92%; ¹H NMR (DMSO-*d*₆): δ 1.21 (s, 3H, CH₃-C2 benzopyran); 1.7 (m, 2H, ArCH₂CH₂); 1.8 (dd, 2H, CH₂, *J* = 4 Hz, *J* = 4.4); 1.95, 2.0, 2.03 (3s, 9H, 3 CH₃ Ar); 2.49 (t, 2H, ArCH₂CH₂); 3.9–4.1 (m, 2H, H-C5 dioxolane); 4.24 (m, 1H, H-C4 dioxolane); 4.77 (d, 1H, H-C5 furanone, *J* = 4 Hz); 5.02 (t, 1H, H-C2 dioxolane); 7.40 (s, 1H, OH 6 benzopyran); 8.5 (sbr, 1H, OH at C3 furanone); 11.35 (br, 1H, OH at C4 furanone). MALDI-TOF MS: m/z 406.9 (M)⁺, C₂₁H₂₆O₈ requires 406.4. Anal. C, H.

11c foam; 109 mg, yield 93%; ¹H NMR (DMSO-*d*₆): δ 1.21 (s, 3H, CH₃-C2 benzopyran); 1.7 (m, 2H, ArCH₂CH₂); 1.8 (dd, 2H, CH₂, *J* = 4 Hz, *J* = 4.4); 1.95, 2.0, 2.03 (3s, 9H, 3 CH₃ Ar); 2.49 (t, 2H, ArCH₂CH₂); 3.9–4.1 (m, 2H, H-C5 dioxolane); 4.24 (m, 1H, H-C4 dioxolane); 4.73 (d, 1H, H-C5 furanone, *J* = 4 Hz); 5.02 (t, 1H, H-C2 dioxolane); 7.42 (s, 1H, OH benzopyran); 8.5 (sbr, 1H, OH at C3 furanone); 11.35 (br, 1H, OH, at C4 furanone). MALDI-TOF MS: m/z 406.9 (M)⁺, C₂₁H₂₆O₈ requires 406.4. Anal. C, H.

11d foam; 106 mg, yield 90%; ¹H NMR (DMSO-*d*₆): δ 1.21 (s, 3H, CH₃-C2 benzopyran); 1.7 (m, 2H, ArCH₂CH₂); 1.8 (dd, 2H, CH₂, *J* = 4 Hz, *J* = 4.4); 1.95, 2.0, 2.03 (3s, 9H, 3 CH₂ Ar); 2.49 (t, 2H, ArCH₂CH₂); 3.9–4.1 (m, 2H, H-C5 dioxolane); 4.24 (m, 1H, H-C4 dioxolane); 4.72 (d, 1H, H-C5 furanone, *J* = 4 Hz); 5.02 (t, 1H, H-C2 dioxolane); 7.42 (s, 1H, OH benzopyran); 8.5 (sbr, 1H, OH at C3 furanone); 11.35 (br, 1H, OH, at C4 furanone). MALDI-TOF MS: m/z 406.9 (M)⁺, C₂₁H₂₆O₈ requires 406.4. Anal. C, H.

4-*O*-Benzyloxy-5*R*-2*S*-(6-benzyloxy-2,5,7,8-tetramethylchroman-2(*R,S*)-yl-methyl)-1,3]dioxolan-4*S*-yl]-3-octadecyloxy-5*H*-furan-2-one (10a,b)- and 4-*O*-benzyloxy-5*R*-2*R*-(6-benzyloxy-2,5,7,8-tetramethylchroman-2(*R,S*)-yl-methyl)-1,3]dioxolan-4*S*-yl]-3-octadecyloxy-5*H*-furan-2-one (10c,d). 2-*O*-Octadecyl-3-*O*-benzyl-ascorbic acid (**8**) (414 mg, 0.8 mmol) was reacted with **6** (540 mg, 1.6 mmol) in the same conditions described above for **9a–d**. After work up the solvent was removed under reduced pressure to give a 1:1:1:1 mixture of the four diastereoisomers. The crude product was purified by silica gel chromatography (hexane:Et₂O 6:4) to give **10a,b** and **10c,d** as inseparable mixtures of the couple of two diastereo-isomers.

10a,b: oil; 230 mg, yield 34%; ¹H NMR (CDCl₃): δ 0.80 (m, 3H, CH₃); 1.2 (s, 3H, CH₃-C2 benzopyran); 1.22–1.26 (m, 32H, alkyl); 1.6–1.8 (m, 2H, ArCH₂CH₂); 1.9 (m, 2H, CH₂); 2.01, 2.08, 2.14 (3s, 9H, 3 CH₃ Ar); 2.5 (t, 2H, ArCH₂CH₂); 3.8–4.0 (m, 4H, -OCH₂ alkyl, CH₂-C5 dioxolane); 4.1–4.3 (m, 1H, H-C4 dioxolane); 4.48 (a) and 4.49 (b) (d×2, 1H, *J* = 4 Hz, H-C5 furanone); 4.61 (d, 2H, CH₂-Ph benzopyran); 5.06–5.08 (m, 1H, H-C2 dioxolane); 5.4 (s, 2H, CH₂-Ph at C4 furanone); 7.1–7.4 (m, 10H, Ph). MALDI-TOF MS: m/z 839.8 (M)⁺, C₅₃H₇₄O₈ requires 839.14.

10c,d: foam; 310 mg, yield 46%; ¹H NMR (CDCl₃): δ 0.80 (m, 3H, CH₃ alkyl); 1.20 (s, 3H, CH₃-C2 benzopyran); 1.22–1.26 (m, 32H, alkyl); 1.6–1.8 (m, 2H, ArCH₂CH₂); 1.9 (m, 2H, CH₂); 2.01, 2.08, 2.14 (3s, 9H, 3 CH₃ Ar); 2.5 (t, 2H, ArCH₂CH₂); 3.8–4.0 (m, 4H, -OCH₂ alkyl, CH₂-C5 dioxolane); 4.1–4.3 (m, 1H, H-C4 dioxolane); 4.50 (c) and 4.52 (d) (d×2, 1H, H-C5 furanone, *J* = 4 Hz); 4.61 (d, 2H, CH₂-Ph benzopyran); 5.08–5.10 (m, 1H, H-C2 dioxolane); 5.4 (s, 2H, CH₂-Ph at C4 furanone); 7.1–7.4 (m, 10H, Ph). MALDI-TOF MS: m/z 840.2 (M + H)⁺, C₅₃H₇₄O₈ requires 839.14.

4-*O*-Hydroxy-5*R*-2*S*-(6-hydroxy-2,5,7,8-tetramethylchroman-2(*R,S*)-yl-methyl)-1,3]dioxolan-4*S*-yl]-3-octadecyloxy-5*H*-furan-2-one (12a,b) and 4-*O*-hydroxy-5*R*-2*R*-(6-hydroxy-2,5,7,8-tetramethylchroman-2(*R,S*)-yl-methyl)-1,3]dioxolan-4*S*-yl]-3-octadecyloxy-5*H*-furan-2-one (12c,d). Compounds **10a,b** (200 mg, 0.24 mmol) and **10c,d** (280 mg, 0.33 mmol) were deprotected by the procedure described above for compounds **9a–d**. After the usual work up and silica gel chromatography purification (CH₂Cl₂:MeOH 9:1), the expected **12a,b** and **12c,d** were obtained in good yields as inseparable mixtures of the of two diastereoisomer.

12a,b: oil; 142 mg, yield 90%; ¹H NMR (DMSO-*d*₆): δ 0.84 (t, 3H, *J* = 6 Hz, CH₃, alkyl); 1.20 (s, 3H, CH₃-C2 benzopyran); 1.22–1.26 (m, 32H, CH₂ alkyl); 1.6–1.8 (m, 2H, Ar-CH₂-CH₂); 1.9 (dd, 2H, *J* = 5 and 4.4 Hz, -CH₂-); 1.95, 1.99, 2.03 (3 s, 9H, 3 CH₃ Ar); 2.44–2.5 (m, 2H, Ar-CH₂-CH₂-); 3.83–3.92 (m, 4H, CH₂-C5 dioxolane, -OCH₂ alkyl); 4.31–4.38 (m, 1H, -CH, H-C4 dioxolane); 4.82 (a) and 4.84 (b) (d, 1H, *J* = 2.2 Hz, H-C5 furanone); 4.96–5.01 (m, 1H, H-C2 dioxolane); 7.40 (sbr, 1H, OH benzopyran); 10.9 (sbr, 1H, OH at C4 furanone). MALDI-TOF MS: m/z 659.8 (M + H)⁺, C₃₉H₆₂O₈ requires 658.9. Anal. C, H.

12c,d: foam; 148 mg, yield 94%; ¹H NMR (DMSO-*d*₆): δ 0.84 (t, 3H, *J* = 6 Hz, CH₃, alkyl); 1.20 (s, 3H, CH₃-C2 benzopyran); 1.22–1.26 (m, 32H, CH₂, alkyl); 1.6–1.8 (m, 2H, Ar-CH₂-CH₂); 1.9 (dd, 2H, *J* = 5 and 4.4 Hz, -CH₂-); 1.95, 1.99, 2.03 (3 s, 9H, 3 CH₃ Ar); 2.44–2.5 (m, 2H, Ar-CH₂-CH₂); 3.83–3.92 (m, 4H, CH₂-C5 dioxolane, -OCH₂ CH₂ alkyl); 4.31–4.38 (m, 1H, -CH-, H-C4 dioxolane); 4.78 (d, 1H, *J* = 2.2 Hz, H-C5 furanone); 4.99–5.01 (m, 1H, -CH-, H-C2 dioxolane); 7.42 (sbr, 1H, OH benzopyran); 11.1 (sbr, 1H, OH at C4 furanone). MALDI-TOF MS: m/z 658.9 (M)⁺, C₃₉H₆₂O₈ requires 658.9. Anal. C, H.

Chemical stability

Solutions of compounds **11a–d** were prepared dissolving an aliquot of compounds in water at pH 3, 5 and 8 to give a final concentration of about 10^{−3} M. The solution was maintained at 37 °C and aliquots were withdrawn every 2 h for the initial 12 h of incubation and successively every 12 h for 4 days. The disappearance of the compounds was monitored by HPLC analysis using the method 1. Conjugates half-lives were from 1 to 3 days.

Molecular modeling

Computational studies have been conducted on a Silicon Graphics Indigo 2 and O2 work stations using the SYBYL²⁰ and SPARTAN²¹ software packages. A systematic conformational search was conducted on compounds 11(a,b) *cis* and 11(c,d) *trans* with SYBYL and all the conformers obtained were energy minimized using MAXIMIN2 (Tripos force field, Powell method²²). Semiempirical quantum-mechanic calculations were performed using the MOPAC (AM1) program²³ under SYBYL. The conformations having closer distances between C5 proton at the furanone ring and C3 proton and C2 methyl at the chromanyl residue were studied with *ab initio* Hartree-Fock methods,²⁴ geometry was optimized with STO-3G basic set to determine the position of the three ring systems.

Biology

Preparation of microsomal membranes

Microsomal membranes were isolated from adult male Wistar rats as previously reported.²⁵ Microsomal protein were determined by Bradford method.

Lipid peroxidation. Lipid peroxidation was initiated by 2-2'-azobis(2-amidinopropane)dihydrochloride (AAPH) as previously described.¹⁰ The reaction was carried out with 25 mM AAPH at 37°C under air for 15 min. Lipid peroxidation was measured by the appearance of malondialdehyde (MDA) formation. MDA was extracted and analyzed by HPLC as indicated.²⁶ Briefly, aliquots of 1 mL of membrane suspension were mixed with 0.5 mL of TBA solution (two parts 0.4% TBA in 0.2 M HCl and one part distilled water) and 0.07 mL of 0.2 BHT in 95% ethanol. Samples were then placed in a 90°C water bath for 45 min. After incubation, the TBA-MDA adduct was extracted with isobutyl alcohol. The isobutanol extract was mixed with methanol (2:1) prior to injection in HPLC system. The column was packed with Supelcosil LC-18 material, 3 µm particle size, in a 15 cm×4.6 mm cartridge format (Supelco, Bellefonte, PA). A 2 cm cartridge precolumn containing 5 µm LC-18 Supelcosil packing was used. The mobile phase was a 1:1 (v:v) mixture of methanol and double distilled water, with the addition of tetrabutyl ammonium dihydrogen phosphate (0.06%, w:v), as an ion pairing reagent. The TBA-MDA adduct was detected by a fluorimeter set at an excitation wavelength of 515 nm and an emission wavelength of 550 nm. At a flow rate of 1 mL/min, the retention time of TBA-MDA adduct was 5 min. MDA concentration was calculated from a calibration curve generated from a peak height of the MDA standard, prepared by acid hydrolysis of 1,1,3,3-tetramethoxypropane.²⁷ The concentration necessary to inhibit MDA formation by 50% (IC₅₀) was calculated from linear plots (concentration versus percent of control) of at least three different concentrations.

Pharmacology

Ischemia-reperfusion in isolated rabbit heart

All experimental protocols were approved by the Animal Care Committee of the University of Milan and were in accordance with the Italian guidelines for the laboratory animals which conform with the European Communities Directive of November 1986 (86/609/EEC).

Male New Zealand White rabbits (BMG-Allevamento, Civitate al Piano, BG, Italy) weighing 2.2–2.5 kg were used for these experiments. The hearts were excised and perfused retrogradely at 37°C through the aorta as previously described by Henry et al.²⁷ and slightly modified by Berti et al.²⁸ The perfusion medium (Krebs Henseleit) contained (in mM): NaCl 118, KCl 2.8, KH₂PO₄ 1.2, CaCl₂ 2.5, MgSO₄ 1.2, NaHCO₃ 25 and glucose 5.5. The pH of the perfusate was 7.4 after a period of equilibration with a 5% CO₂ and 95% O₂ gas mixture, and the rate of perfusion was maintained at 20 mL/min with a roller pump (Minipuls 3, Gilson, Villiers-Le Bel, France). Coronary perfusion pressure (CPP) and left ventricular pressure (LVP) were measured with two HP-1280C pressure transducers (Hewlett-Packard, Waltham, MA, USA) connected with a Hewlett-Packard dynograph (HP-7754A). LVP was recorded with a polyethylene catheter (with a small latex balloon on the top) inserted in the left ventricular cavity. The balloon was filled slowly with saline until left ventricular end-diastolic pressure (LVEDP) stabilised in the range of 4–6 mmHg. Left ventricular developed pressure (LVDP; peak left ventricular systolic pressure minus LVEDP) was also evaluated. The hearts were electrically paced at a frequency of 180 beats/min with rectangular impulses (1 ms duration, voltage 10% above threshold) by a Grass stimulator (mod. S-88; Grass Instr., Quincy, MA, USA). CPP of 55–60 mmHg was obtained with a flow of 20 mL/min (preischemic period). Ischemia was induced by reducing the flow rate to 1 mL/min for 40 min (ischemic period; CPP = 12–14 mmHg). A normal flow rate (20 mL/min) was then restored, and the perfusion was continued for 20 min (reperfusion period). The compound under investigation, 11a–d was tested in groups of five hearts each. In particular, 11a–d (100–300 µM) was perfused through the hearts for a period of 30 min before reduction of coronary flow.

Creatine kinase determination

The heart perfusates were collected every 150 s in an ice-cooled beaker before flow reduction and during reperfusion and the activity of creatine kinase (CK) was evaluated according to the method of Bergmeyer et al.²⁹ by a specific kit (Amersham-Italia, MI, Italy). The amount of the enzyme was determined on a Lambda16-spectrophotometer (Perkin-Elmer Italia, Monza, MI, Italy) and expressed as mU/min/g/wet tissue.

Data analysis

In all experiments, differences between control and treated groups were analyzed for statistical significance using a one-way analysis of variance (ANOVA) and

Tukey–Kramer test for multiple comparisons. A value of $p < 0.05$ was considered significant. In all figures, results are expressed as mean \pm standard error of the mean (SEM). The areas under the curve (AUCs) were assessed by using a computerized program Microcal Origin.³⁰

Acknowledgements

This work was supported by the University of Ferrara, Italy.

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